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Desiccation tolerance of the invasive alga starry stonewort (*Nitellopsis obtusa*) as an indicator of overland spread risk

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ABSTRACT

Human-assisted transport via recreational boats and trailers is thought to account for most contemporary movement of aquatic invasive species (AIS) among lakes. The ability of invasive macrophytes to survive out of water, that is, their desiccation tolerance, is an important indicator of capacity for spread to new water bodies through overland transport. Invasion by the alga starry stonewort (*Nitellopsis obtusa* [Desv. in Loisel.] J. Groves; Characeae) in North America is likely driven via overland transport, but little is known regarding its ability to remain viable out of water. We conducted laboratory and outdoor experiments to evaluate desiccation tolerance of starry stonewort propagules, including single stem fragments, small and large clumps of fragments, and bulbils (asexual reproductive structures). Propagules were removed from water for 15 min to 5 d to identify desiccation thresholds. Fully desiccated fragments and clumps lost ~90% of their original mass and bulbils > 60% of original mass. Based on the most conservative estimates from our experiments, starry stonewort was no longer viable at 2 h for single fragments, 24 h for small (5-g) clumps, 4 d for large (45-g) clumps, and 4 h for bulbils. Overall, starry stonewort appears less tolerant of desiccation than other invasive macrophytes that have been evaluated, which comprise vascular plants rather than Characean algae. Widely adopted guidance that boats should dry on land for 5 d to prevent AIS spread should suffice to prevent most starry stonewort spread between water bodies, given that boaters comply with inspection protocols and remove large, readily detected clumps.

Key words: bulbil, charophyte, invasive species, macroalga, overland transport, viability

INTRODUCTION

Spread of aquatic invasive species (AIS) via overland transport is a major driver of new invasions (Johnstone et al. 1985, Buchan and Padilla 1999, Johnson et al. 2001). Although overland transport can occur via animals (Reynolds et al. 2015, Green 2016), human-assisted transport via recreational boats and trailers likely accounts for a great majority of recent AIS dispersal events (Johnson and Carlton 1996, Buchan and Padilla 1999). Overland dispersal of aquatic species occurs when a propagule is able to remain viable until introduction to a new water body (Vander Zanden and Olden 2008). Hence, for an aquatic invasive plant species to establish in a new water body, a propagule must arrive with the ability to sprout, continue growth, or regenerate from living tissue. Removing plant material from watercraft is effective for reducing spread risk, but recreational boaters often fail to check and clean their boats upon leaving a water body (Rothlisberger et al. 2010, Cimino and Strecker 2018). Even when watercraft are inspected and cleaned, a small amount of plant material typically remains (Rothlisberger et al. 2010). Hence, the ability of an aquatic invasive plant to survive out of water, i.e., desiccation tolerance, is an important indicator of its ability to spread to new water bodies. Invasive macrophytes differ in their ability to tolerate desiccation (Barnes et al. 2013, Bruckerhoff et al. 2015); thus, individual species need to be examined to determine spread risk.

Starry stonewort (*Nitellopsis obtusa* [Desv. in Loisel.] J. Groves; Characeae) is an invasive macroalga native to Europe and Asia. The first documented record of starry stonewort in North America is from the St. Lawrence River in Quebec, Canada, in 1974 (Karol and Sleith 2017). Starry stonewort has since been discovered in eight states in the United States and in two Canadian provinces (Kipp et al. 2018). Starry stonewort can produce substantial biomass (Schloesser et al. 1986, Glisson et al. 2018) and form tall, dense beds (Sher-Kaul et al. 1995, Pullman and Crawford 2010, Boissezon et al. 2018). This abundant growth can hinder recreation, reduce native macrophyte abundance and richness, and potentially have further ecological consequences, such as altering water chemistry or fish habitat (Pullman and Crawford 2010, Brainard and Schulz 2017, Larkin et al. 2018). Starry stonewort's ecological niche in North America appears broad and could potentially expand under future climate change scenarios (Escobar et al. 2016, Romero-Alvarez et al. 2017, Muthukrishnan et al.

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2018, Sleith et al. 2018). Once starry stonewort is established in a water body, sustained control of an infestation may be difficult (Larkin et al. 2018, Glisson et al. 2018).

To guide strategic efforts to prevent starry stonewort spread, it is important to understand the capacity of starry stonewort to invade new water bodies via overland transport. The association of starry stonewort occurrences with boat launches, high dock density, and nearby infested lakes (Sleith et al. 2015, Midwood et al. 2016) is consistent with overland transport via watercraft and trailers being the dominant means of spread. Desiccation during overland transport is a critical bottleneck for the spread of AIS. However, there are currently no studies on the desiccation tolerance of starry stonewort. Understanding how long starry stonewort can remain viable out of water, and the corresponding distance boaters can travel in that time, is needed to better evaluate spread risk, target interventions (e.g., watercraft inspection and early detection surveillance), and prevent spread. Moreover, knowledge of starry stonewort desiccation tolerance can determine whether recommended drying times for watercraft are sufficient for starry stonewort. Species-specific information for starry stonewort is essential because past studies of macrophyte desiccation tolerance have been performed on vascular plants, which are evolutionarily and anatomically distinct from Characean algae (Raven et al. 2005), and thus may be poor analogues for estimating drying thresholds.

We conducted controlled experiments, both outdoors and in the laboratory, to examine desiccation tolerance of starry stonewort. We tested how long starry stonewort propagules could remain viable out of water by examining single fragments (segments of stem-like thalli), small clumps of fragments, and large clumps of fragments, anticipating that desiccation would be slower for greater amounts of material. We also tested desiccation tolerance of starry stonewort bulbils—asexual reproductive structures formed belowground that are a key means of spread (Larkin et al. 2018). Because only male starry stonewort has been found in North America (Larkin et al. 2018), sexual reproduction is not a known means of spread and oospores were not evaluated in this study. We evaluated all material over a wide range of drying times (from 15 min to 5 d). We then estimated the duration of time required for starry stonewort propagules to become fully desiccated and no longer viable. We conducted two experiments to examine desiccation tolerance of starry stonewort: 1) a climate-controlled laboratory experiment, and 2) an outdoor experiment subject to natural weather variation. We conducted the outdoor experiment first and made minor changes for the laboratory experiment to improve precision of our results. As the methods for the laboratory experiment largely comprise those for the outdoor experiment, we present the methods for the laboratory experiment first.

MATERIALS AND METHODS

Laboratory desiccation experiment

Assessment of viability. To determine desiccation tolerance of starry stonewort fragments in the laboratory experiment, we used three approaches to assess viability. First, we evaluated the tendency of starry stonewort to lose mass following removal from water, that is, *mass loss following desiccation*, by comparing mass loss of samples removed at increasing drying-time endpoints to those of fully desiccated dry controls. Additionally, we examined the rate of *mass loss following desiccation*. Next, we determined the ability of starry stonewort to recover lost mass via rehydration, that is, *mass recovery following rehydration*, by comparing unrecovered mass of rehydrated samples from each drying-time endpoint to both dry controls (maximum unrecovered mass) and never-dried wet controls (minimum unrecovered mass). Last, to supplement the previous two approaches, we determined the ability of starry stonewort to recover its physical condition (i.e., color and turgor) via rehydration, that is, *physical recovery following rehydration*, by visually comparing rehydrated samples to wet controls. We used the most conservative drying-time endpoint determined by the first two approaches as the time at which starry stonewort was no longer viable, and used the third approach to confirm this result. We did not assess viability of starry stonewort fragments using direct indicators of growth (e.g., thallus growth or rhizoid formation), as it has proven difficult to grow and maintain starry stonewort cultures from thalli. Mass loss following desiccation has been shown to be a significant predictor of, and therefore, a reliable proxy for macrophyte viability (Evans et al. 2011, Barnes et al. 2013, Bickel 2015). We directly assessed the viability of starry stonewort bulbils following desiccation by comparing sprouting of bulbils removed at each drying-time endpoint to wet controls.

Experimental setup. We collected live starry stonewort and bulbils for the laboratory experiment on October 1, 2017 from Lake Koronis in Minnesota (Stearns and Meeker counties) using a rake attached to a telescoping pole. Collected material was stored in lake water during transport and overnight prior to experiments beginning the following day (all material was used within 24 h of collection). Experiments were conducted at the Minnesota Aquatic Invasive Species Research Center's Containment Laboratory (MCL) on the University of Minnesota's St. Paul campus.

Fragments. We examined starry stonewort fragments of three different size classes: single fragments, small clumps, and large clumps. Single fragments included three nodes (an apical node and two nodes along the thallus), were 10–20 cm long, and weighed ~0.5 g (mean wet weight = 0.49 ± 0.01 g [1 SE]). We gently aggregated starry stonewort fragments into small and large clumps of ~5 g (4.84 ± 0.08 g) and ~45 g (45.00 ± 0.26 g), respectively. Small and large clumps were ovoid; small clumps were approximately 6 × 3 cm and large clumps 9 × 6 cm.

We allowed starry stonewort samples to dry under controlled conditions and then removed samples at 11 drying-time endpoints (time treatments): 0.25, 0.5, 1, 2, 4, 6, 12, 24, 48, 72, and 120 h. We included a negative control (never dried, i.e., wet control) and a positive control (dried to constant mass, i.e., dry control) for each size class. For each of the 13 total treatments (11 time treatments and wet and dry controls), we used 15 replicates for single fragments ($N = 195$) and 10 replicates for small and large clumps ($N =$

130 each). We desiccated dry controls in paper bags in a drying oven at 60 C for 3 d.

We conducted the laboratory desiccation experiment in the MCL inside a 3 × 3 m mesh tent with a solid floor to maintain containment. We weighed all samples prior to the start of the experiment to determine initial weight and then attached samples to strings strung between two upright PVC frames within the tent. Samples from each time treatment were randomly assigned to two strings each and strings were separated by a height of 30–35 cm. We attached single fragments to strings with a paper clip fastened to the section of the thallus below the third node (similiar to Bruckerhoff et al. 2015) and small and large clumps using cable ties fastened around the center of each clump. Mean temperature in the MCL during the experiment was 24.0 ± 0.17 C and mean relative humidity (RH) was 43.85 ± 1.05%. Temperature and RH were similar to those used in other laboratory-based macrophyte desiccation studies (Jerde et al. 2012, Mcalarnen et al. 2012, Barnes et al. 2013).

At each drying-time endpoint, we removed samples from strings, recorded their dried weight to determine *mass loss following desiccation*, and immediately placed them in dechlorinated water in glass jars. We recorded the dried weight of dry controls following removal from the drying oven and also placed them in water-filled jars. Wet controls were placed in water-filled jars immediately after initial weighing. Jars containing the rehydrated samples were randomly placed on a lab bench and kept under low-light conditions for 6 h (photosynthetically active radiation [PAR] = 37 μmol $m^{-2}s^{-1}$). We then pulled samples from jars and removed excess water by gently shaking single fragments for 5 s and placing small and large clumps in a fine-mesh strainer for 5–10 s. Lastly, we weighed all samples and recorded their rehydrated weights to determine *mass recovery following rehydration*.

After weighing rehydrated samples, we retained a subset to evaluate *physical recovery following rehydration* using six 24-h samples and six wet controls for each size class. We examined samples from only the 24-h treatments to provide a confirmation of the other two approaches, *mass loss following desiccation* and *mass recovery following rehydration*, while balancing the logistical constraints of examining samples from all treatments. We placed the samples in jars filled with 5 cm of a 50 : 50 potting soil : sand mix and water, covered the jars with fine mesh, and placed them in clear tanks filled with water to 10 cm above the tops of the jars. Water was continuously circulated through the tanks and ∼50% of water volume was replaced each day. After 3 wk, we removed the jars and visually inspected each sample for the presence of green, turgid thalli, as an indicator of viability (following Barnes et al. 2013 for *Ceratophyllum demersum*, and Baniszewski et al. 2016 for *Hydrilla verticillata*).

Bulbils. We separated bulbils from rhizoids of starry stonewort collected from Lake Koronis and selected those that appeared robust and were large enough to observe easily (mean wet weight = 0.02 ± 0.01 g [1 SE]). We used 12 bulbils as replicates for each of the 11 time treatments and wet and dry controls ($N = 156$). We placed bulbils into 50-mm petri dishes on a table next to the tent used for fragments and randomly assigned petri dishes to time treatments. We desiccated dry controls in paper coin envelopes in a drying oven at 60 C for 3 d.

At each drying-time endpoint, we removed bulbils from petri dishes, weighed them (all 12 bulbils for each treatment were weighed together for a single treatment weight), and immediately placed them into 4-oz. glass jars filled with 2 cm of a 50 : 50 potting soil : sand mix and water. Bulbils were gently pushed into the sediment until fully covered. Each jar contained four bulbils from a given treatment (three jars per treatment). Jars containing bulbils were covered with fine mesh and placed into three clear plastic tanks filled with water to 10 cm above the tops of the jars. We recorded the dried weight of dry controls following removal from the drying oven and placed them in jars in the same manner as time treatments. Wet controls were placed in jars immediately after initial weighing. We randomized the order of jars in tanks such that each of the three tanks contained one jar from each time treatment and wet and dry control. We maintained tanks under a 14 h/10 h light/dark schedule (PAR = 37 μmol $m^{-2}s^{-1}$ at the water's surface). Mean water temperature in tanks during the experiment was 16.9 C.

We checked bulbils for sprouting every 3–7 d for a total of 8 wk (56 d), which previous research had shown to be sufficient for most bulbils to sprout under similar conditions (Glisson et al. 2018). A bulbil was considered sprouted when a green shoot emerged from the bulbil. Sprouted bulbils were removed from jars to avoid duplicate counting. At the end of the experiment, we were unable to recover all bulbils that we had initially placed in jars. Unrecovered bulbils likely broke apart or decomposed over the course of the experiment and were thus considered nonviable (following Glisson et al. 2018).

Outdoor desiccation experiment

Assessment of viability. To determine desiccation tolerance of starry stonewort fragments in the outdoor experiment, we used two approaches to assess viability. First, we determined *mass loss following desiccation* by comparing mass loss of samples removed at increasing drying-time endpoints to those of 72-h treatments, which we assumed to be fully desiccated. Next, we determined *mass recovery following rehydration* by comparing unrecovered mass of rehydrated samples from each drying-time endpoint to both 72-h treatments that served as dry controls (maximum unrecovered mass), and never-dried wet controls (minimum unrecovered mass). We used the most conservative drying-time endpoint determined by these approaches as the time at which starry stonewort was no longer viable. To assess viability of starry stonewort bulbils following desiccation, we used a chemical stain (following Gottschalk and Karol 2020, this issue; see the following) to compare viability of bulbils from each drying-time endpoint to those in the 72-h treatments.

Fragments. The methods for the outdoor desiccation experiment were generally similar to those used in the laboratory experiment; only methods that differed from the laboratory experiment are described here. We collected live starry stonewort and bulbils for the outdoor experiment on August 7, 2017 from Lake Koronis. Single fragments

weighed ~0.5 g (mean = 0.58 ± 0.02 g [1 SE]), small clumps ~5 g (5.64 ± 0.09 g), and large clumps ~20 g (20.41 ± 0.19 g). We used eight drying-time endpoints (time treatments): 1, 2, 4, 6, 12, 24, 48, and 72 h. We employed only a wet control and we used 10 replicates for each treatment ($N = 90$ for each size class).

We conducted the outdoor desiccation experiment on a paved surface outside the MCL within the same mesh tent used in the laboratory experiment. We weighed all samples initially and then randomly assigned and attached them to strings, separated by a height of 40 cm, tied to PVC frames within the tent. Temperature and relative humidity fluctuated over the course of the experiment (Appendix). It rained several times during the experiment and samples were not protected from getting wet; however, all time treatments ≤ 24 h were removed prior to any rain. At each drying-time endpoint, we removed samples from strings, recorded their dried weight to determine *mass loss following desiccation*, and placed them in water in glass jars for 6 h under low-light conditions (PAR = 37 μmol $m^{-2}s^{-1}$). Wet controls were placed in water-filled jars immediately after initial weighing. We then pulled samples from jars, removed excess water, and weighed all samples and recorded rehydrated weight to determine *mass recovery following rehydration*.

Bulbils. We used 10 bulbils as replicates for each of the eight time treatments and wet controls ($N = 90$). We placed bulbils into 50-mm petri dishes on a table within the tent and haphazardly assigned petri dishes to time treatments. At each drying-time endpoint, we removed bulbils from petri dishes and immediately placed them into 4-oz. glass jars filled with water. Jars were randomly placed on a lab bench and kept under low-light conditions for 6 h (PAR = 27 μmol $m^{-2}s^{-1}$).

To determine bulbil viability, we used a staining compound commonly used for assessment of vascular plant tissue viability: 2,3,5-triphenyl-tetrazolium chloride (TZ). In live cells, TZ is reduced to formazan, a reddish compound that is not water soluble; this results in live cells being stained red (Parker 1953). Staining with TZ is commonly used to assess angiosperm seed and tissue viability, has previously been used to assess algae viability (Nam et al. 1998, Calomeni et al. 2014), and has been effective to determine viability of starry stonewort bulbils (following Gottschalk and Karol 2020, this issue). Following rehydration, we placed all bulbils from each treatment into amber glass jars with 25 ml of 0.1% TZ solution. Jars containing bulbils were stored in a refrigerator for 18–24 h, after which bulbils were examined under a dissecting scope for signs of red staining indicative of live cells. Starry stonewort bulbils are composed of multiple cells; hence, we considered bulbils with red stain visible on at least one cell to be viable.

Statistical analysis

Fragments. All analyses were conducted using R statistical software, version 3.4.3 (R Development Core Team 2017). Analyses were identical between the laboratory experiment and outdoor experiment, except that 8 time treatments were used in the outdoor experiment versus 11 in the laboratory experiment, and the 72-h treatments in the outdoor experiment were used as proxies for dry controls. Lastly, only the first two approaches described below, *mass loss following desiccation* and *mass recovery following rehydration*, were used to assess viability in the outdoor experiment.

To determine *mass loss following desiccation*, we compared the proportion of mass lost following desiccation for each time treatment ([initial weight – dried weight]/initial weight) to dry controls. We used analysis of variance (ANOVA) to examine differences among treatments, with proportion of mass lost as the response variable and treatment (12 levels : 11 time treatments and dry control) as the predictor variable. We performed separate analyses for single fragments, small clumps, and large clumps. We then used Tukey's honest significant differences (Tukey's HSD) to compare each time treatment to the dry control. This allowed us to identify the time point(s) when starry stonewort in our time treatments no longer significantly differed from fully desiccated controls.

To determine the rate of *mass loss following desiccation*, we used nonlinear least squares (NLS) regression to fit a three-parameter asymptotic exponential function to our data. We chose this function because we expected starry stonewort fragments in our experiment to reach an asymptote at which increased drying time does not lead to further mass loss, and because it has been effectively employed in previous macrophyte desiccation experiments (Bickel 2015). We used the equation $y = a - be^{-cx}$, where a is the asymptote, $a - b$ is the intercept, and c is the rate constant. Starting values are required for NLS models; we provided a starting value of 1 for both a and b. To determine a starting value for c, we used x and y values for a point likely to fall along the line of best fit and rearranged the equation above to solve for c (Crawley 2007). We fit NLS models for single fragments, small clumps, and large clumps, with the proportion of mass lost following desiccation as the response variable, and time (in minutes) as the continuous predictor variable. Hence, we did not include controls for this analysis. We determined starting values for c using mean mass loss (y) at 30 min, 12 h, and 48 h (x) for single fragments, small clumps, and large clumps, respectively. Each NLS model estimated an intercept, asymptote, and a rate constant. We considered starry stonewort fully desiccated when the proportion of mass lost predicted from the model was within 1 SE of the asymptote. We compared rate constants for each size class and considered these significantly different if their 95% confidence intervals (CI) did not overlap.

Next, to determine *mass recovery following rehydration*, we compared the proportion of mass not recovered following rehydration (i.e., unrecovered mass) for each time treatment ([initial weight – rehydrated weight]/initial weight) to wet and dry controls. The two controls served as minimum (wet control) and maximum (dry control) benchmarks for unrecovered mass. The same ANOVA and Tukey's HSD analyses described above were repeated for this analysis (for single fragments, small clumps, and large clumps) with proportion of unrecovered mass as the response variable and treatment (13 levels: 11 time treatments, wet control, and dry control) as the predictor variable. This allowed us to

identify time point(s) when unrecovered mass in rehydrated starry stonewort was significantly greater than wet controls and as much as (i.e., not significantly different from) fully desiccated dry controls.

Last, to determine *physical recovery following rehydration*, we visually compared rehydrated 24-h treatments for single fragments, small clumps, and large clumps to wet controls. We scored each sample (consisting of either a single fragment, a small clump, or a large clump) as containing either ≥ 1 green, turgid fragment(s) (viable), or no green, turgid fragment(s) (nonviable). We scored and photographed each sample to document its condition (images available upon request).

Bulbils. We determined the viability of starry stonewort bulbils following desiccation in the laboratory experiment by identifying the time point(s) when bulbils no longer sprouted and when fewer time treatment bulbils sprouted than did wet control bulbils. We analyzed bulbil-sprouting data with a generalized linear model (GLM) with binomial errors and logit link to determine the time point(s) when significantly fewer time treatment bulbils sprouted than wet control bulbils. We used the proportion of bulbils sprouted as the response variable (expressed as counts of sprouted and unsprouted bulbils with $n = 12$ for each treatment) and treatment (13 levels: 11 time treatments, wet control, and dry control) as the predictor variable. We also examined the proportion of bulbil mass lost following desiccation to estimate the mass loss associated with no further sprouting.

RESULTS AND DISCUSSION

Laboratory desiccation experiment

Fragments. In the laboratory experiment, starry stonewort fragments in fully desiccated dry controls lost $\geq 90\%$ of their mass: 90% for single fragments, 91% for small clumps, and 91% for large clumps (Figure 1). Following rehydration, dry controls regained $\geq 38\%$ of that lost mass: 55% for single fragments, 50% for small clumps, and 38% for large clumps (Figure 2).

Based on *mass loss following desiccation*, single fragments were fully desiccated (i.e., the proportion of mass lost no longer significantly differed from dry controls) at the 2-h time point, small clumps at the 24-h time point, and large clumps at the 72-h time point (Figure 1).

The rate of *mass loss following desiccation* significantly differed by size class, with single fragments having the greatest rate of mass loss, followed by small clumps and then large clumps (Table 1, Figure 3). Based on rate constant and asymptote estimates from NLS models, single fragments were fully desiccated at 1 h 25 min, small clumps at 20 h 4 min, and large clumps at 3 d 23 h 57 min (Table 1, Figure 3).

Based on *mass recovery following rehydration*, single fragments could no longer recover from desiccation (i.e., the proportion of unrecovered mass was significantly greater than wet controls, but not significantly different from dry controls) by the 30-min time point, small clumps by the 12-h time point, and large clumps by the 72-h time point (Figure 2).

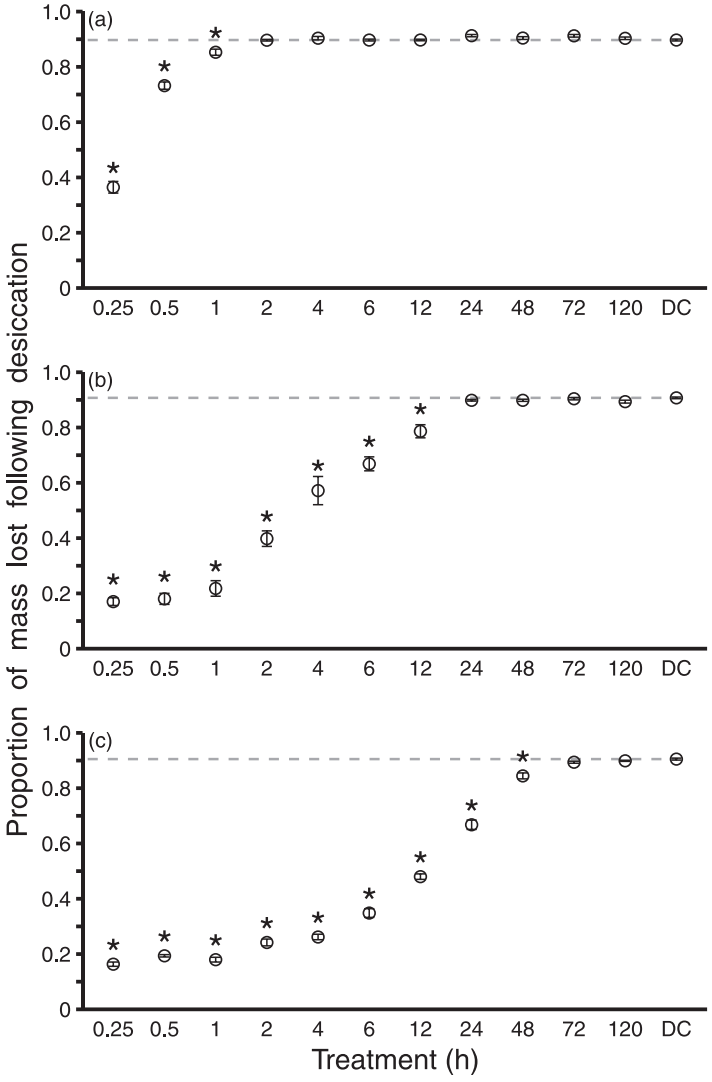

Figure 1. Proportion of starry stonewort mass lost following desiccation at each drying-time endpoint (treatment) in the laboratory experiment for (a) single fragments, (b) small clumps, and (c) large clumps. Points and error bars are means ±1 SE. Dry controls (DC) were fully desiccated samples. Dashed gray line is the proportion of mass lost in the dry controls. An asterisk indicates a significant difference between that time treatment and the dry control.

In terms of *physical recovery following rehydration*, none of the 24-h single-fragment or small-clump treatments contained viable material, whereas all wet controls did. For large clumps, five of six (83%) 24-h treatment jars and all six wet control jars contained viable material.

Based on the three approaches used to assess viability, starry stonewort in the laboratory desiccation experiment was no longer viable at 2 h for single fragments, 24 h for small clumps, and 4 d for large clumps (Table 2).

Bulbils. After 8 wk, ≥ 1 bulbil(s) sprouted in the wet control (9 sprouted bulbils) and 15-min (5), 30-min (11), 1-h (9), and 2-h (1) treatments (Figure 4a). We observed no sprouting in any time treatments ≥ 4 h or the dry controls. For the treatments in which bulbils did sprout, sprouting in the 2-h treatment was significantly lower than the wet control ($P = 0.0062$). Hence,

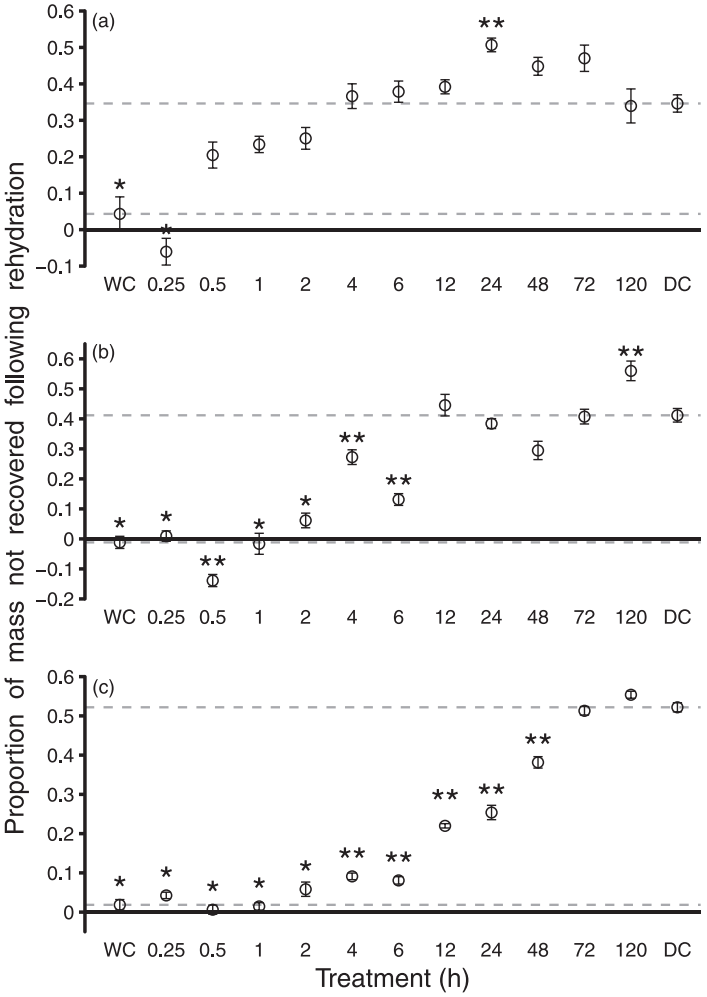

Figure 2. Proportion of starry stonewort mass not recovered following rehydration at each drying-time endpoint (treatment) in the laboratory desiccation experiment for (a) single fragments, (b) small clumps, and (c) large clumps. Points and error bars are means ±1 SE. Wet controls (WC) were samples that were never dried, and dry controls (DC) were fully desiccated samples. Dashed gray lines are the proportion of mass not recovered in the dry controls (top line) and wet controls (bottom line). One asterisk indicates a significant difference between that time treatment or control and the dry control; two asterisks indicate a significant difference between that time treatment and both the wet control and dry control. Treatments significantly different from wet controls, but not significantly different from the dry controls (no asterisks), were considered unable to recover from desiccation.

starry stonewort bulbils in the laboratory desiccation experiment were no longer viable at 4 h. Starry stonewort bulbils in the dry controls lost 74% of their mass; no bulbils sprouted when ≥ 59% of their mass was lost (Figure 4b).

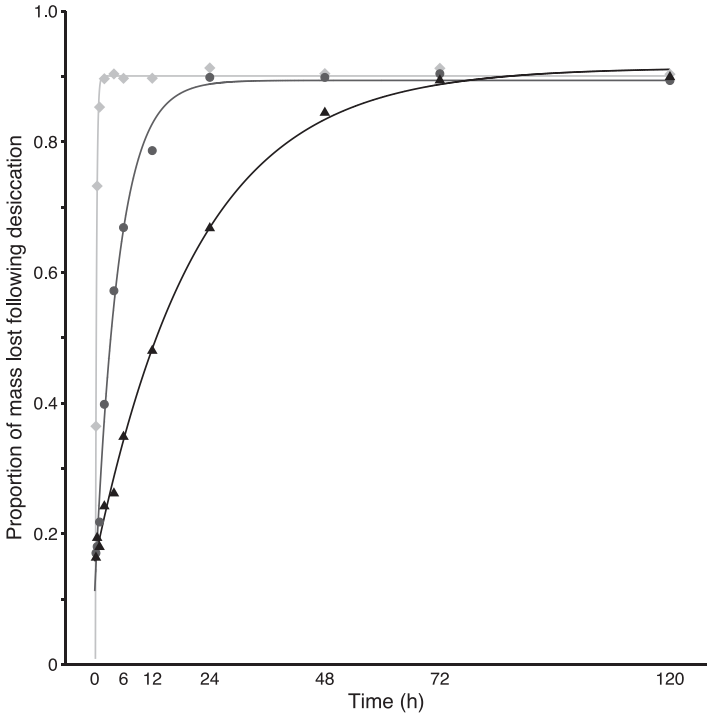

Figure 3. Rates of starry stonewort mass loss over time in the laboratory desiccation experiment for single fragments (light gray; diamonds), small clumps (gray; circles), and large clumps (black; triangles). Rate of mass loss was determined by a three-parameter asymptotic exponential function fit by nonlinear least squares regression.

Outdoor desiccation experiment

In the outdoor experiment, starry stonewort fragments in the fully desiccated 72-h treatments lost ≥ 93% of their mass: 94% for single fragments, 93% for small clumps, and 93% for large clumps (Figure 5). Following rehydration, the 72-h treatments regained ≥ 36% of that lost mass: 36% for single fragments, 50% for small clumps, and 48% for large clumps (Figure 6).

Based on *mass loss following desiccation*, single fragments were fully desiccated (i.e., the proportion of mass lost no longer significantly differed from the 72-h treatments) at the 1-h time point, small clumps at the 2-h time point, and large clumps at the 6-h time point (Figure 5). For both small and large clumps, the 48-h treatment significantly differed from the 72-h treatment (Figure 5); however, the 48-h treatment was removed after it had rained (see Appendix).

Based on *mass recovery following rehydration*, for single fragments, unrecovered mass of rehydrated starry stonewort was significantly greater than wet controls in the 1-h

Table 1. Estimates of rate constants (±1 SE), 95% confidence intervals (CI) for rate constants, and asymptotes (proportion of mass lost ±1 SE) from nonlinear least-squares regression models for starry stonewort desiccation in the laboratory experiment. Desiccation threshold (asymptote – 1 SE) is the proportion of mass lost at which the material was considered nonviable. Different letters indicate significant differences.

Sample	Rate Constant	95% CI	Asymptote	Desiccation Threshold
Single fragment	0.0733 (0.0036)[a]	0.066–0.081	0.901 (0.003)	0.898
Small clump	0.0034 (0.0002)[b]	0.003–0.004	0.894 (0.013)	0.881
Large clump	0.0008 (0.0000)[c]	0.001–0.001	0.913 (0.008)	0.905

Table 2. Results from laboratory and outdoor desiccation experiments on starry stonewort, including the experiment, type of sample examined, mean weight of the samples (±1 SE), the approach used to assess viability, and time at which starry stonewort was determined to be no longer viable.

Experiment	Sample Type	Mean Weight (g)	Approach	Time At Which No Longer Viable
Laboratory	Single fragment	0.49 (0.01)	Mass loss	2 h
			Rate of mass loss	1 h 25 min
			Mass recovery	30 min
			Physical recovery	24 h
	Small clump	4.84 (0.08)	Mass loss	24 h
			Rate of mass loss	20 h 4 min
			Mass recovery	12 h
			Physical recovery	24 h
	Large clump	45.00 (0.26)	Mass loss	72 h
			Rate of mass loss	3 d 23 h 57 min
			Mass recovery	72 h
			Physical recovery	> 24 h
	Bulbils	0.02 (0.01)	Sprouting	4 h
Outdoor	Single fragment	0.58 (0.02)	Mass loss	1 h
			Mass recovery	1 h
	Small clump	5.64 (0.09)	Mass loss	2 h
			Mass recovery	6 h
	Large clump	20.41 (0.19)	Mass loss	6 h
			Mass recovery	6 h
	Bulbils	NA	TZ staining	1 h

treatment but, unexpectedly, not in the 2-h treatment; all single-fragment time treatments $\geq$ 1 h did not significantly differ from the fully desiccated 72-h treatment (Figure 6). For small clumps, unrecovered mass of rehydrated starry stonewort was significantly greater than the wet controls in all treatments $\geq$ 1 h. Except for the 12-h treatment, small-clump time treatments $\geq$ 6 h did not significantly differ from the fully desiccated 72-h treatment (Figure 6). For large clumps, unrecovered mass of rehydrated starry stonewort was significantly greater than the wet controls in all time treatments $\geq$ 1 h. Large-clump time treatments $\geq$ 6 h did not significantly differ from the fully desiccated 72-h treatment, except for the 48-h treatment that was exposed to rain (Figure 6, Appendix).

In general, for unrecovered mass (i.e., *mass recovery following rehydration*), there was greater variance within treatments (Figure 6) and less clarity regarding the time at which starry stonewort was no longer viable compared to the examination of mass lost (i.e., *mass loss following desiccation*: Figure 5). Variation among treatments could be explained by changes in weather conditions over the course of the experiment (e.g., rain prior to removal of the 48-h treatments), whereas variation within or among treatments could be explained by inconsistency in removing excess water from samples before weighing. Given the overall pattern of responses to desiccation, we believe the inconsistent data for unrecovered mass (2-h treatment for single fragments and 12-h treatment for small clumps; Figure 6) reflects the latter explanation—variation in how these samples were handled in the experiment—rather than actual starry stonewort response to desiccation. Hence, we chose to consider the first treatment for which the unrecovered mass criteria were met (1-h treatment for single fragments and 6-h treatment for small clumps) as the time point to assess viability using the *mass recovery following rehydration* approach.

Based on the two approaches to assess viability, starry stonewort in the outdoor desiccation experiment was no longer viable at 1 h for single fragments, 6 h for small clumps, and 6 h for large clumps (Table 2). We observed red staining from the reduction of TZ on 6 of 10 wet control bulbils (60%) and none of the time treatment bulbils ($\geq$ 1 h), indicating bulbils were no longer viable at 1 h.

Synthesis

Compared to other aquatic invasive plant species that have been evaluated, starry stonewort appears to have lower desiccation tolerance. Single fragments of starry stonewort in both the laboratory and outdoor experiments lost viability more quickly than similarly sized fragments of Eurasian watermilfoil (*Myriophyllum spicatum*; Evans et al. 2011), Carolina fanwort (*Cabomba caroliniana*; Bickel 2015), curly-leaf pondweed (*Potamogeton crispus*; Bruckerhoff et al. 2015), and hydrilla (*Hydrilla verticillata*; Baniszewski et al. 2016) in passive desiccation studies (i.e., studies that did not simulate wind exposure associated with overland travel). The duration of time that single fragments of starry stonewort remained viable was similar to that reported for other invasive macrophytes subjected to active drying (i.e., fan-dried at 7.5 mph), including Eurasian watermilfoil, Carolina fanwort, and curly-leaf pondweed (Jerde et al. 2012, Barnes et al. 2013). We would expect starry stonewort to desiccate substantially faster if actively dried, as wind

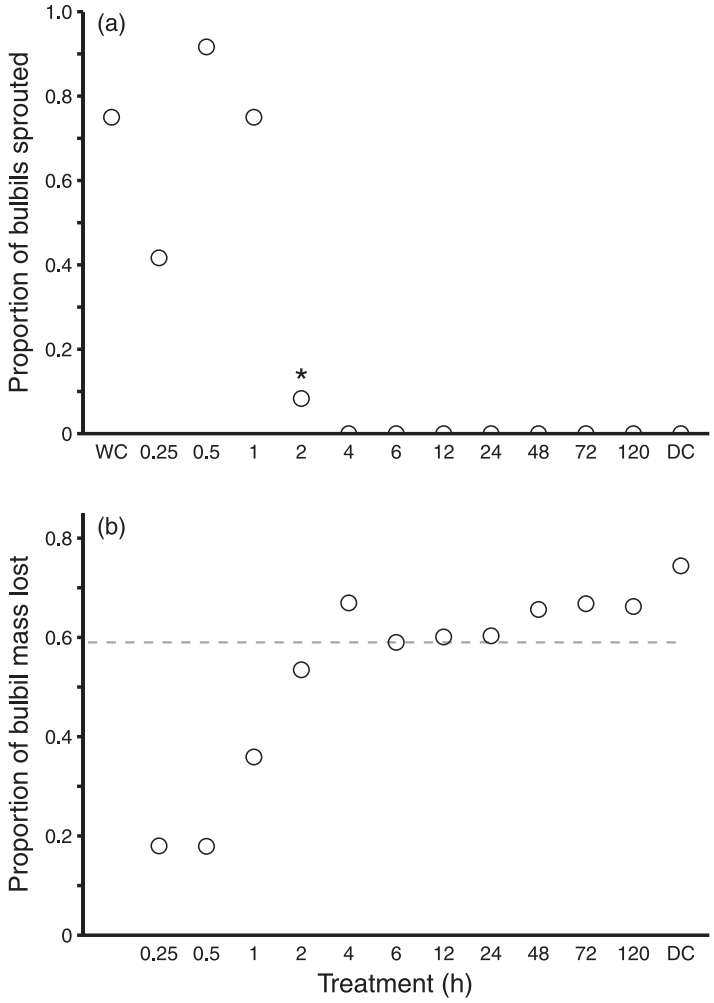

Figure 4. Response of starry stonewort bulbils to desiccation in the laboratory experiment, including proportion of (a) bulbils sprouted, and (b) bulbil mass lost following desiccation at each drying-time endpoint (treatment). Wet controls (WC) were bulbils that were never dried, and dry controls (DC) were fully desiccated bulbils. Dashed gray line is the lowest proportion of bulbil mass lost at which bulbils no longer sprouted (6-h treatment). Wet control bulbils were weighed prior to the experiment only, and hence, not included in (b). For (a), an asterisk indicates a significant difference between that time treatment and the wet control.

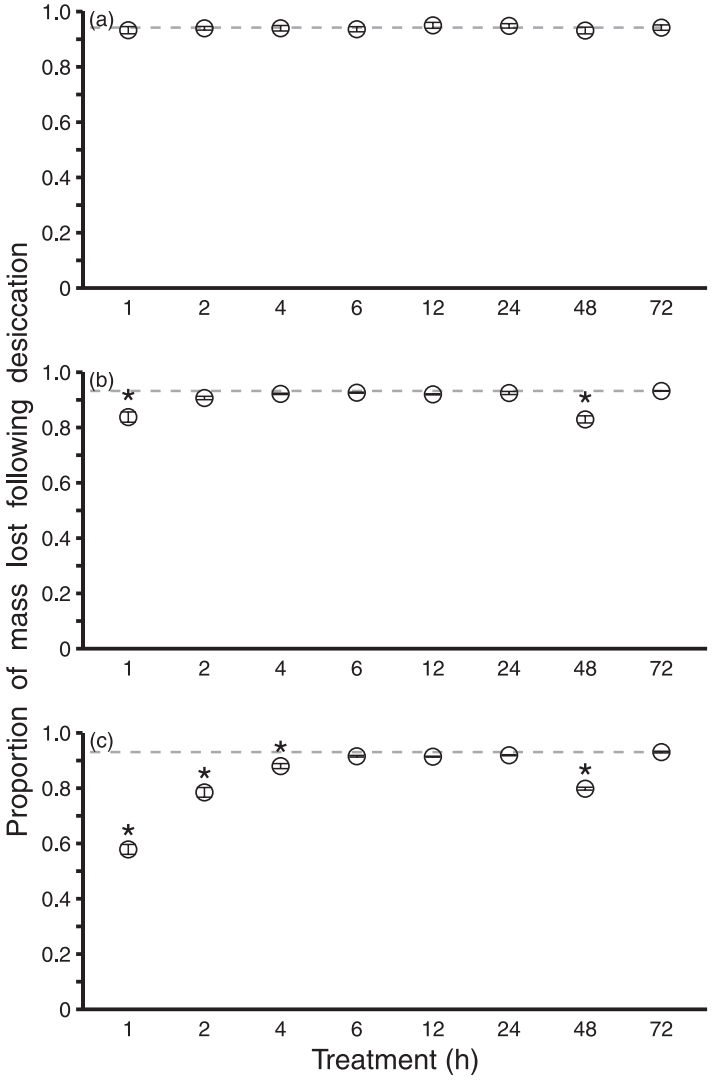

Figure 5. Proportion of starry stonewort mass lost following desiccation at each drying-time endpoint (treatment) in the outdoor desiccation experiment for (a) single fragments, (b) small clumps, and (c) large clumps. Points and error bars are means ±1 SE. Dashed gray line is the proportion of mass lost in the 72-h treatment. An asterisk indicates a significant difference between that time treatment and the 72-h treatment.

speed can greatly increase desiccation rate (Bickel 2015). Regardless, passively dried single fragments of starry stonewort in our laboratory experiment became fully desiccated faster than five other aquatic plant species exposed to 7.5-mph wind speed by Barnes et al. (2013), and fragments in our outdoor experiment desiccated faster than all nine species examined in their study.

Comparison of starry stonewort desiccation to other species should be interpreted in light of macrophyte desiccation experiments varying in the type (e.g., apical vs. basal), length, node number, and mass of fragments evaluated, all of which can influence desiccation rate and viability (Mcalarnen et al. 2012; Bickel 2015, 2017; Li et al. 2015). These factors may affect comparability among studies and species. Our single fragments likely had lower mass than fragments used in similar studies with other species, but they were similar in length to fragments of Eurasian watermilfoil (Jerde et al. 2012, Mcalarnen et al. 2012, Barnes et al. 2013) and similar in length and number of nodes to fragments of Carolina fanwort (Bickel 2015) used in other studies. We also used apical stems in our experiment and these stems can have greater viability and slower desiccation rates than basal stems (Mcalarnen et al. 2012). Thus, the estimates for desiccation times and loss of viability we determined may be conservative; that is, basal starry stonewort fragments may have indicated even lower desiccation tolerance.

As an alga, starry stonewort has morphological characteristics that likely make fragments less tolerant to desiccation than other macrophytes. Namely, starry stonewort lacks the multicellular tissues found in vascular plants (i.e., vascular, dermal, and ground tissues; Raven et al. 2005).

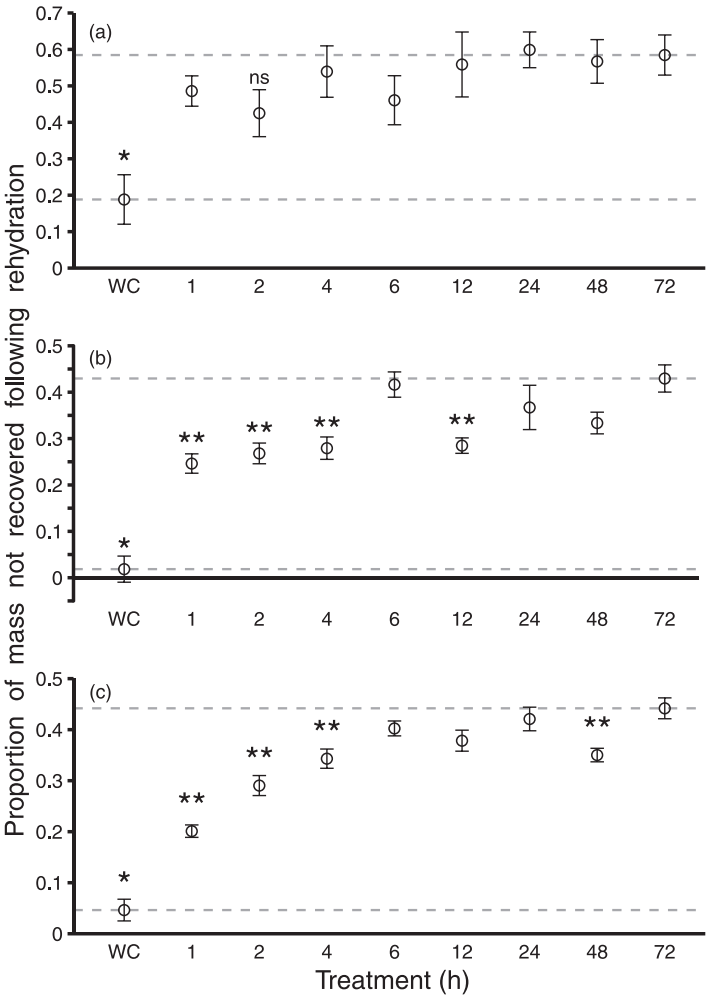

Figure 6. Proportion of starry stonewort mass not recovered following rehydration at each drying-time endpoint (treatment) in the outdoor desiccation experiment for (a) single fragments, (b) small clumps, and (c) large clumps. Points and error bars are means ±1 SE. Wet controls (WC) were samples that were never dried. Dashed gray lines are the proportion of mass not recovered in the 72-h treatments (top line) and the wet control (bottom line). One asterisk indicates a significant difference between that time treatment or control and the 72-h treatment; two asterisks indicate a significant difference between that time treatment and both the wet control and the 72-h treatment; ns indicates a time treatment that was not significantly different from the wet control or the 72-h treatment. Treatments significantly different from wet controls, but not significantly different from the 72-h treatment (no asterisks), were considered unable to recover from desiccation.

The presence of these multicellular tissues in the vascular macrophytes typically evaluated in similar desiccation studies (e.g., Barnes et al. 2013) likely allows these plants to retain moisture longer. Starry stonewort has only a single elongated cell between nodes (up to 24 cm long; Steudle et al. 1977, Bharathan 1983). The branchlets of starry stonewort, which form whorls at each node (similar in appearance to the leaves of a vascular plant), are also primarily composed of single elongated cells (Bharathan 1983, Boissezon et al. 2018). Hence, the bulk of starry stonewort cells have very high surface area–to–volume ratios; this characteristic, along with the high water permeability of internodal starry stonewort cells (Yoshioka and Takenaka 1979), likely facilitates rapid desiccation following removal from water. Moreover, the single elongated cells of starry stonewort are easily broken or crushed when handled, releasing the cells' cytoplasm and rendering them nonviable. We handled fragments carefully in our experiments, but it is likely that fragments would encounter greater force when snagged on a motor or trailer.

Starry stonewort desiccated approximately twice as quickly in the outdoor experiment than the laboratory experiment. Although the large clumps in the outdoor experiment weighed an average of 25 g (56%) less than those in the laboratory experiment, they became fully desiccated in $\leq 10\%$ of the time. The temperature on the first day of the outdoor experiment—when starry stonewort of all size classes was fully desiccated—was only slightly higher than the mean temperature in the laboratory experiment (25.2 versus 24.0 C). Relative humidity was also only slightly higher on the first day of the outdoor experiment (47.0 versus 44.9%). Thus, it is unlikely that temperature and humidity account for the differences in desiccation tolerance we observed between the outdoor and laboratory experiments. Rather, exposure to wind and sunlight (Appendix), that is, conditions more representative of those encountered during overland transport, are likely the main factors that lowered desiccation tolerance in the outdoor experiment. Future controlled experiments that vary weather conditions (e.g., wind, humidity, temperature, and sunlight exposure) could help determine the role of these factors on starry stonewort desiccation tolerance and the ability to survive overland transport under realistic scenarios.

Single fragments of starry stonewort desiccated quickly, but clumps of starry stonewort remained viable for far longer. This is consistent with other desiccation studies that have examined clumps or coils of macrophyte stems. For example, coiling of Eurasian watermilfoil stems increased desiccation times from 24 to 72 h (Bruckerhoff et al. 2015), and clumping of Carolina fanwort stems increased desiccation times from 9 to 42 h (Bickel 2015); we observed a similar pattern for starry stonewort. This is unsurprising, given the capacity of larger clumps to retain more moisture because of lower surface area–to–volume ratio.

Starry stonewort bulbils dried quickly in our experiments; bulbils were no longer viable within 1 h in the outdoor experiment and 4 h in the laboratory experiment. These times are substantially less than those reported for the asexual reproductive structures (turions) of curly-leaf pondweed, which can survive > 4 wk out of water (Bruckerhoff et al. 2015) or seeds of Eurasian watermilfoil, which can survive > 8 mo out of water (Standifer and Madsen 1997). It is not surprising that starry stonewort bulbils dried more quickly than these reproductive structures, as the regenerative cells of bulbils lack the hard protective coatings found in seeds or turions of other aquatic plants (Bharathan 1987). Starry stonewort bulbils sprout quickly and in high proportion (this study; Bharathan 1987, Glisson et al. 2018), but their susceptibility to desiccation may limit their potential to spread and form large infestations. However, we did not examine desiccation

tolerance of starry stonewort bulbils embedded in clumps, attached to rhizoids, or associated with sediment. Bulbils under such conditions can survive out of water longer (12–24 h; Gottschalk and Karol 2020, this issue), likely because of buffering of moisture loss.

CONCLUSION

Our findings provide estimates for how long starry stonewort fragments and bulbils can remain viable out of water, a key indicator of overland spread risk. These estimates can guide prevention protocols and guidelines for watercraft leaving starry stonewort–infested water bodies. The relatively low desiccation tolerance we observed is encouraging in terms of preventing the spread of starry stonewort via overland transport. The smallest pieces of starry stonewort—that is, single fragments and individual bulbils most likely to be missed during visual inspection—quickly lost viability. Boaters traveling to multiple lakes on the same day may unintentionally move viable single fragments and bulbils to new water bodies, but it is unlikely that these propagules would survive if a boat were kept out of water for a day or more. Although small and large clumps remained viable for longer, the size of these clumps makes them easier to locate during inspection—particularly the large clumps that retained viability longest. Furthermore, our findings from the outdoor experiment demonstrate that even these large clumps of starry stonewort can dry rapidly under warm, sunny conditions with natural air movement. Fragments and clumps would likely desiccate even faster if exposed to the wind experienced during vehicle travel (Bickel 2015). For the masses of starry stonewort and conditions evaluated in this study, adherence to the 5-d drying time recommended by state agencies such as the Minnesota Department of Natural Resources (Minnesota Department of Natural Resources [DNR] 2018a) should ensure that starry stonewort is no longer viable before entering another water body.

Despite these findings of starry stonewort's relatively low desiccation tolerance, this species is nevertheless expanding in its invaded range (Kipp et al. 2018, Minnesota DNR 2018b). Although its spread may be constrained by lower desiccation tolerance, opportunities for spread are clearly occurring. Rare, long-distance dispersal events can have a substantial influence on the spread of invasive plant species (Higgins and Richardson 1999). Such opportunities could arise when boaters do not comply with requirements to inspect and remove plants from watercraft and trailers (Rothlisberger et al. 2010, Cimino and Strecker 2018). Fragments and bulbils missed during inspection that are protected from moisture loss or left submerged in residual water will likely remain viable much longer, providing increased opportunity for spread. For example, starry stonewort propagules caught between a boat hull and carpeted bunk trailer are likely to escape detection and, due to buffering of moisture loss, could potentially remain viable for substantially longer periods than the material observed in our experiments. We did not examine algal material protected from moisture loss in our experiments, but this could be examined in future work for a better understanding of starry stonewort's ability to spread via overland transport. Moreover, there is still a chance that stems left on a boat or trailer that become fully desiccated and appear nonviable, are in fact viable (e.g., Evans et al. 2011). Thus, relying solely on the tendency of starry stonewort, or any AIS, to dry out quickly is insufficient for spread prevention. Visual inspection and hand removal, however, are effective means to remove invasive aquatic plants from watercraft (Rothlisberger et al. 2010) and should be used at a minimum when leaving and entering a water body. If such inspection protocols are followed, starry stonewort masses larger than those evaluated in this study (and hence, potentially able to remain viable longer) should be readily detected by boaters. More aggressive strategies such as steam treatments may also be effective at reducing starry stonewort viability and spread (Gottschalk and Karol 2020, this issue).

Given that starry stonewort infestations can be large and difficult to manage (Glisson et al. 2018), it is crucial to inspect and remove this alga before it can be transported to a new water body. Maps of invasion risk for starry stonewort have been developed in the upper Midwest United States (Muthukrishnan et al. 2018) and these can be used with the findings reported here to focus watercraft inspection efforts. These efforts, combined with outreach programs and compliance by individual boaters, are vital for preventing the spread of starry stonewort.

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Appendix. Temperature, relative humidity, wind speed, and precipitation during the starry stonewort outdoor desiccation experiment conducted August 8–11, 2017. Data are from the August 2017 local climatological report for Minneapolis, MN, and were collected at the Minneapolis–St. Paul International Airport (KMSP; 44°52′N; 93°13′W; National Oceanographic and Atmospheric Administration 2017).

Day	Hour	Temperature (C)	Relative Humidity (%)	Wind Speed (mph)	Precipitation (cm)
August 8, 2017	9	23.3	56	6	0
	12	27.2	39	11	0
	15	28.3	36	8	0
	18	27.2	38	6	0
	21	23.9	52	8	0
	24	21.1	61	0	0
	Mean	25.2	47	6.5	Daily total = 0.00
August 9, 2017	3	20.0	65	7	0
	6	18.9	68	5	0
	9	19.4	71	10	0.03
	12	18.3	87	8	0.25
	15	19.4	87	10	0.03
	18	19.4	84	8	0
	21	19.4	81	5	0
	24	17.2	87	17	0.48
	Mean	19.0	78.8	8.8	Daily total = 1.45
August 10, 2017	3	16.1	93	5	0
	6	16.7	90	9	0
	9	18.3	81	8	0
	12	21.7	66	15	0
	15	21.7	73	11	0.02
	18	21.7	63	13	0
	21	18.9	73	6	0
	24	16.7	84	5	0
	Mean	19.0	77.9	9.0	Daily total = 0.03
August 11, 2017	3	15.0	90	6	0
	6	15.0	93	5	0
	9	20.6	66	7	0
	12	25.0	45	11	0
	Mean	18.9	73.5	7.25	Daily total = 0.00
	Mean	20.4	70.3	8.1	Total = 0.81